Isoprostanes constrict human radial artery by stimulation of thromboxane receptors, Ca^{2+} release, and RhoA activation

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Objectives: Radial artery vasospasm remains a potential cause of early graft failure after coronary bypass graft surgery, despite pretreatment with α-adrenergic or calcium channel blockers. We examined the roles of isoprostanes and prostanoid receptors selective for thromboxane A_2 in the vasoconstriction of human radial arteries.

Methods: Human radial arterial segments were pretreated intraoperatively with verapamil/papaverine or nitroglycerine/phenoxybenzamine, or not treated. In the laboratory, we measured isometric contractions in ring segments, vasoconstriction in pressurized segments, and changes in $[Ca^{2+}]$ and K^+ currents in single cells.

Results: Although phenoxybenzamine eliminated adrenergic responses, the isoprostane 15-F_{2t}-IsoP and 2 closely related E-ring molecules (15-E_{1t}-IsoP and 15-E_{2t}-IsoP) still evoked powerful contractions; 15-E_{2t}-IsoP was approximately 10-fold more potent than the other 2 agents. Responses were mediated through thromboxane receptors because they were sensitive to ICI-192605. Furthermore, they were sensitive to the Rho-kinase inhibitors Y-27632 or H-1152 (both 10^{-5} mol/L) or to cyclopiazonic acid (which depletes the internal Ca^{2+} pool), but not to nifedipine. In single cells, 15-E_{2t}-IsoP elevated $[Ca^{2+}]_i$ and suppressed K^+ current.

Conclusions: Isoprostanes accumulate after coronary artery bypass graft surgery, yet none of the currently available antispasm treatments for radial artery grafts is effective against isoprostane-induced vasoconstriction. It is imperative that more specific treatment strategies be developed. We found that isoprostane responses in radial arteries are mediated by prostanoid receptors selective for thromboxane A_2 with activation of Rho-kinase and release of Ca^{2+}. Pretreatment of radial artery grafts with Rho-associated kinase inhibitors may potentially reduce postoperative graft spasm. Clinical studies to test this are indicated.

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The long-term benefits of arterial conduits in coronary artery bypass graft (CABG) surgery are well established.[1,2] Radial artery (RA) grafts have been used frequently because of their versatile applications, easy handling, and relatively simple harvesting with low incidences of postoperative complications.[3-5] However, the relatively more muscular nature of the RA grafts makes them more susceptible to mechanical and pharmacologic stimulations,[6] which usually result in vasoconstriction (although the actual postsurgical incidence of this is unclear). In its most severe form, the entire RA graft can close off as the result of graft spasm. The precise mechanism of RA graft spasm is still not well understood: The strategies developed to treat it have largely been empirical and based on hypothetic extrapolations from other tissues, and have not succeeded in preventing RA spasm com-

Abbreviations and Acronyms

CABG = coronary artery bypass graft
KCl = potassium chloride
NE = norepinephrine
RA = radial artery
ROCK = Rho-associated kinase
TP receptors = prostanoid receptors selective for thromboxane A_2

pletely. Therefore, there is a need to better understand the mechanisms underlying perioperative RA spasm.

The CABG operation is essentially an ischemia-reperfusion injury that is known to set the stage for the production of free radicals that attack membrane lipids, leading in part to the production of metabolites known as isoprostanes.[7] The latter can be produced while the lipids are in free form, as well as when they are still esterified within the membrane (to be released even weeks later by phospholipases). In fact, isoprostanes are widely used as markers of oxidative stress. Several groups have now documented their accumulation in the blood after CABG.[8-10] However, our group and many others have shown isoprostanes to be powerfully bioactive;[7,11] for example, they are powerful vasoconstrictor agents acting through prostanoid receptors selective for thromboxane A_2 (TP receptors). Here, we compared the responsiveness of human radial arteries with 3 different isoprostanes and used a variety of pharmacologic tools to characterize the signaling pathway(s) by which they act. We examined the effects of several different isoprostanes on tension and vessel diameter in whole tissues, as well as cytosolic $[Ca^{2+}]$ and K^+ currents in single cells. Altogether, we document powerful excitatory effects of the isoprostanes on the human RA. Concurrently, we considered the relative efficacies of 2 different pharmacologic strategies currently used within the operative suite aimed at preventing vasospasm after CABG surgery.

Materials and Methods

Preparation of Arterial Tissues

All experimental procedures were approved by the ethics committee of St Joseph's Healthcare and Hamilton Health Sciences.

RA tissues were harvested as pedicles (with veins and periarterial fat) using the traditional open technique and treated by immersion for 30 minutes at room temperature in Ringer's lactate containing either verapamil plus papaverine (3.25 and 0.25 mg/mL, respectively) or phenoxybenzamine plus nitroglycerine (2.0 and 0.2 mg/mL, respectively). A third set of tissues were set aside without undergoing any such pretreatment (control). RA tissues that were not used in the surgical procedure were then transported on ice to the laboratory where the loosely adherent adipose and connective tissues were removed and the arterial vessels were cut into ring segments approximately 3 to 4 mm long, and then used immediately or stored overnight for use the following day.

Tissue Bath Technique

Radial arterial rings were mounted in standard 2.5-mL muscle baths containing Krebs–Ringer buffer (see below for composition); a stretch of 1 g was imposed perpendicularly to the axis of the lumen, and isometric contractions were recorded as we have described.[12] These were equilibrated for 1 hour by challenging with 60 mmol/L KCl 3 times, after which responsiveness to adrenergic stimuli and/or to U-46619 (a thromboxane A_2 analogue) was assessed. Where indicated, some tissues were left in the muscle baths overnight (at 37°C and with continuous bubbling) for the same assessment the following day.

Video-monitored Perfusion System

Radial arterial segments were kept overnight in Dulbecco's Modified Eagle Medium at 37°C. The segments were cannulated at both ends and mounted in a video-monitored perfusion system (Living Systems Instrumentation Inc, Burlington, Vt). The artery was bathed in an 8-mL chamber containing Krebs–Ringer buffer (continuously bubbled) on the stage of an inverted microscope and superfused externally at a rate of 2 mL/min by Krebs–Ringer buffer (at 37°C). Distal and proximal pressures were monitored, and the mean arterial pressure was controlled distally to 85 mm Hg by a pressure servocontrol system. Images were acquired using a digital camera (30 frames/sec) and passed through a video dimension analyzer that derives overall vessel diameter on a frame-by-frame basis. Arteries were equilibrated for 1 hour in Krebs-Ringer's buffer (37°C), replaced at 15-minute intervals, challenged twice with 60 mmol/L KCl, and then challenged with isoprostanes 15-F_{2t}-IsoP and 15-E_{2t}-IsoP before and after treatment with pharmacologic blockers.

Single Cell Studies

Individual myocytes were dissociated from human RA rings using collagenase, elastase, and papain/dithiothreitol, as we described previously.[13,14] Cytosolic concentrations of $[Ca^{2+}]_i$ were recorded using a custom-built confocal microscope, and membrane currents were recorded using the standard patch-clamp electrophysiologic technique, both as described previously.[13-16]

Solutions and Chemicals

Krebs–Ringer buffer contained 116 mmol/L of NaCl, 4.2 mmol/L of KCl, 2.5 mmol/L of $CaCl_2$, 1.6 mmol/L of NaH_2PO_4, 1.2 mmol/L of $MgSO_4$, 22 mmol/L of $NaHCO_3$, and 11 mmol/L of D-glucose, bubbled to maintain the pH at 7.4. L-NNA (10^{-4} mol/L) and indomethacin (10^{-5} mol/L) were also added to prevent the generation of nitric oxide and cyclo-oxygenase metabolites of arachidonic acid, respectively. Chemicals were obtained from Sigma Chemical Company (St Louis, Mo), except for U46619 (Cayman Chemical Company; Ann Arbor, Mich), ICI 192605 (Tocris; Ellisville, Mo), H-1152 (Calbiochem; distributed by VWR Canlab, Mississauga, Ontario), and fluo-4 AM and pluronic acid (Molecular Probes; distributed by Invitrogen Canada, Burlington, Ontario). Pharmacologic tools were prepared in distilled water (norepinephrine [NE]; phenylephrine; phentolamine, H-1152), eth-

anol, or dimethyl sulfoxide (isoprostanes, U46619; nifedipine; cyclopiazonic acid; Y-27632; ICI 192,605).

Data Analysis

All data are reported as mean ± standard error of the mean; n refers to the number of patients. Statistical comparisons were made using 2-way analysis of variance with Newman–Keuls post hoc test.

Results

Responsiveness to Nonspecific Constrictor Stimulus

Potassium chloride (KCl) acts primarily by depolarizing the membrane and triggering voltage-dependent Ca^{2+} influx.[17] By using tissues that had been treated intraoperatively with various pharmacologic regimens, we compared KCl-evoked contractions on the day they were collected and after overnight incubation in the muscle baths with maintained warming and bubbling. Figure 1 summarizes the mean magnitudes of those responses. The responses in the verapamil/papaverine-treated tissues were significantly smaller than in the control, whereas those of the phenoxybenzamine/nitroglycerine-treated tissues were not. Also, no statistically significant difference was observed between day 1 and day 2 within a given group.

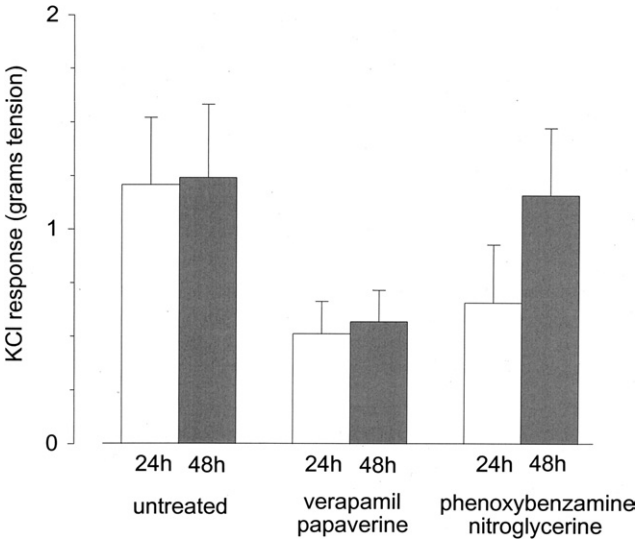

Figure 1. Responsiveness to KCl. Contractile responses to KCl (60 mmol/L) were assessed in ring segments treated intraoperatively with verapamil plus papaverine (n = 12) or phenoxybenzamine plus nitroglycerine (n = 18), or not at all (n = 11); see "Materials and Methods" for details. Responses were assessed on the same day the tissue was excised (*open bars*) and in the same tissues on the following day (*filled bars*). *KCl,* Potassium chloride.

Responsiveness to Receptor-specific Stimuli

We also assessed the responsiveness of the tissues to an α-adrenoreceptor agonist (NE) or to several isoprostanes (15-F_{2t}-IsoP and 2 closely related E-ring molecules 15-E_{1t}-IsoP and 15-E_{2t}-IsoP) added in 10-fold increments in cumulative fashion. Tissue strips that received no pharmacologic treatment in the surgical suite exhibited dose-dependent increases in tension in response to NE (Figure 2, *A*). The maximal response to NE was approximately equivalent in magnitude to that of the KCl responses evoked in the same tissues. Likewise, in tissues that had been treated in the surgical suite with verapamil and papaverine, NE evoked concentration-dependent contractions that were not significantly different in magnitude from the untreated tissues (Figure 2, *A*). Tissues pretreated with the α-adrenoceptor antagonist phenoxybenzamine, however, exhibited no appreciable response to NE (Figure 2, *A*). Nonetheless, the latter did exhibit powerful concentration-dependent tension responses to all 3 isoprostanes. Figure 2, *B* summarizes the mean concentration-response relationships so obtained (n = 5). All 3 isoprostanes evoked contractions that were approximately double the amplitude of the KCl-evoked response. With respect to potency, the tissues were approximately 10-fold more responsive to 15-E_{2t}-IsoP than to the other 2 compounds, with a half-maximally effective concentration of 3×10^{-7} mol/L. Because isoprostanes generally exert their actions through TP receptors, we tested the effects of the TP-receptor antagonist ICI 192605 (10^{-6} mol/L; n = 5) or GR32191B (10^{-6} mol/L; n = 3): Responsiveness to the most potent isoprostane, 15-E_{2t}-IsoP, was abrogated by GR32191 and abolished by ICI 192605 (Figure 2, *C*; n = 5). This responsiveness was not affected, however, by supplementation of α-adrenoceptor blockade using phentolamine (10^{-5} mol/L; n = 4). Likewise in pressurized arteries, both 15-E_{2t}-IsoP and 15-F_{1t}-IsoP caused concentration-dependent decreases in diameter (Figure 3; n = 4), with 15-E_{2t}-IsoP showing greater potency than 15-F_{2t}-IsoP (statistically significant different at 10^{-7} mol/L), which were blocked by ICI 192,605 (Figure 4; n = 4).

Pharmacologic Sensitivity of 15-E_{2t}-IsoP–evoked Vasoconstrictor Responses

Next, we examined the sensitivity of 15-E_{2t}-IsoP–evoked responses to pretreatment with a variety of pharmacologic blockers; the data are summarized in Figures 4 (pressurized arterial segments) and 5 (isometric contractions). Responses to 15-E_{2t}-IsoP were completely unaffected by nifedipine (10^{-5} mol/L; n = 7; blocker of voltage-dependent Ca^{2+}-channels). Cyclopiazonic acid (10^{-5} mol/L; n = 7; depletes the internal Ca^{2+} pool), on the other hand, partially reduced the magnitude of the responses, particularly those evoked by micromolar concentrations of isoprostane (statistically significant only at 10^{-5} mol/L 15-E_{1t}-IsoP). Isoprostanes may increase the Ca^{2+} sensitivity of the contractile apparatus via activation of ROCK.[12,18] To assess the involvement of this

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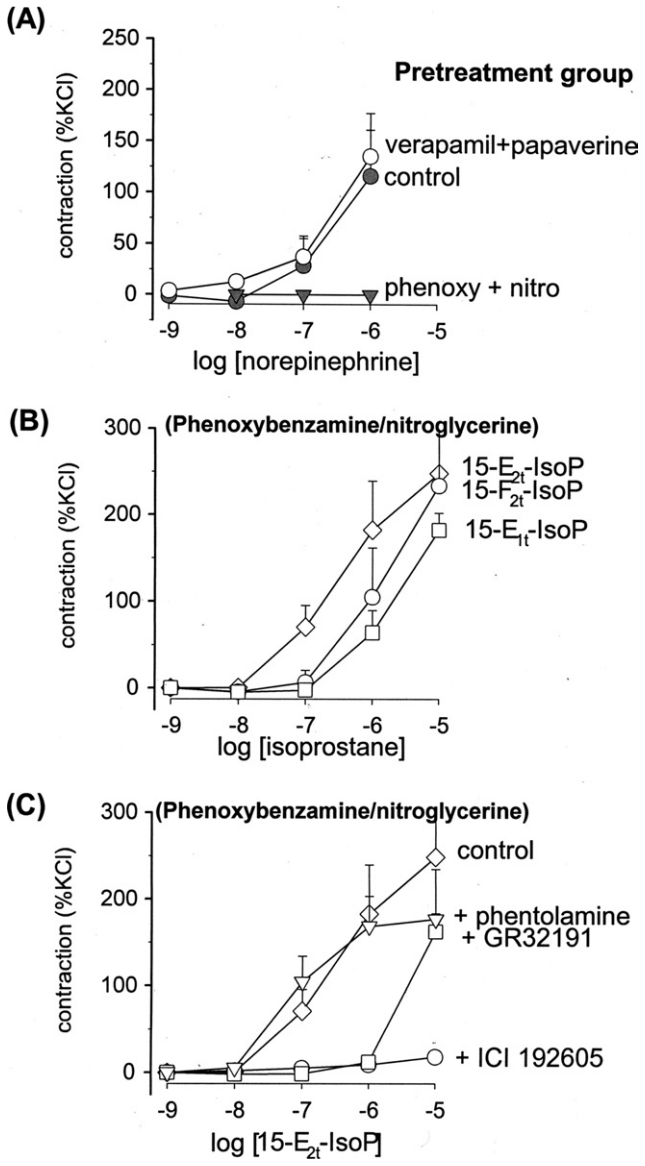

Figure 2. Responsiveness to receptor-specific stimuli. Mean concentration-response relationships for NE or 3 different isoprostanes (15-F_{2t}-IsoP, 15-E_{1t}-IsoP, and 15-E_{2t}-IsoP) in human RA. A, NE-evoked responses in tissues that did not receive intraoperative pretreatment (*filled circles*) or those pretreated with verapamil/papaverine (*open circles*) or phenoxybenzamine plus nitroglycerine (*filled triangles*). B, Isoprostane-evoked responses in tissues pretreated intraoperatively with phenoxybenzamine/nitroglycerine. C, Effects of the TP-receptor blockers ICI 192605 (10^{-6} mol/L; n = 5) or GR 32191B (10^{-6} mol/L; n = 3) or the α-adrenoceptor blocker phentolamine (n = 4) against responses to 15-E_{2t}-IsoP in phenoxybenzamine/nitroglycerine-treated tissues. *KCl,* Potassium chloride.

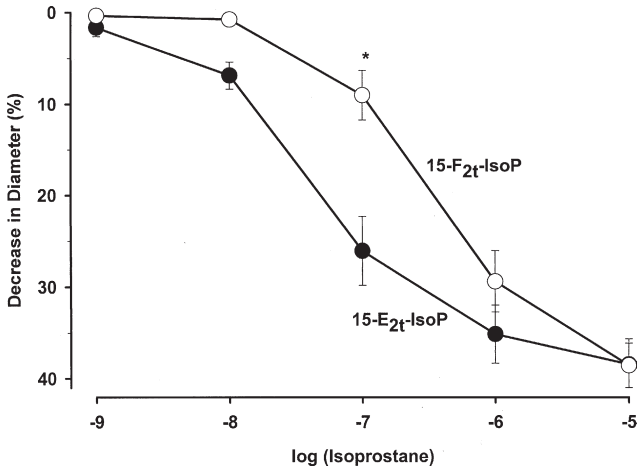

Figure 3. Isoprostane-evoked constrictions under auxotonic conditions. Mean changes in arterial diameter of human RA pressurized to 85 mm Hg and challenged with increasing concentrations of either 15-F_{2t}-IsoP or 15-E_{2t}-IsoP, added in cumulative fashion (n = 4).

pathway, we used 2 different ROCK inhibitors, Y-27632 and H-1152. Both significantly attenuated isoprostane-induced contractions (Figure 4).

Fluorimetry

Human radial arterial myocytes loaded with the Ca^{2+}-indicator dye fluo-4 and studied using confocal fluorimetric techniques exhibited a substantial increase in fluorescence above baseline on application of 15-E_{2t}-IsoP (10^{-5} mol/L). Figure 6, *A* illustrates a representative example, and Figure 6, *B* compares the mean responses to isoprostane or to caffeine in 11 cells (n = 4).

Patch-clamp Electrophysiology

Depolarizing pulses (from −60 to + 50 mV, 10-mV increments) from a holding potential of −70 mV evoked outwardly rectifying K^+ currents at potentials greater then −40 mV. A representative recording is given in Figure 7. Application of 15-E_{2t}-IsoP (10^{-5} mol/L, through a micropuffer) did not increase the holding current but did statistically significantly suppress the K^+ currents: at a potential of +30 mV, these were reduced by 22.6% ± 6.1% (n = 7).

Discussion

Before their use in CABG surgery, human RA segments are routinely pretreated perioperatively with pharmacologic tools selected for their ability to inhibit perio- or postoperative vasoconstriction. We compared the responsiveness of human RA tissues pretreated using 2 common intraoperative regimens for their responsiveness to isoprostanes. We

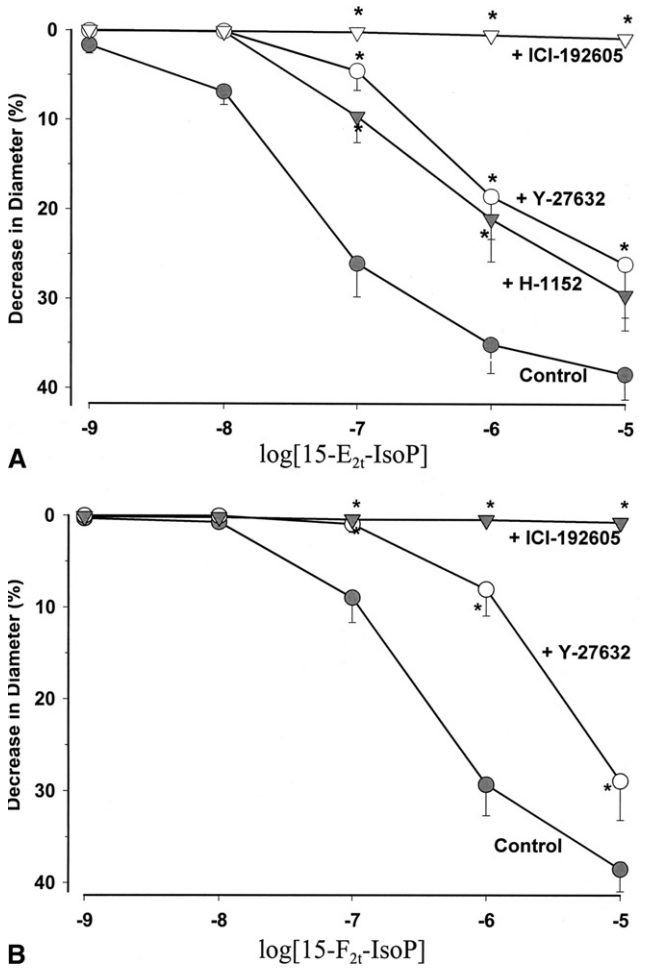

Figure 4. Pharmacologic characterization of the role of ROCK in isoprostane-evoked responses. A, Mean percentage change in diameter to increasing concentrations of 15-E_{2t}-IsoP (n = 11) in the absence or presence of Y-27632 (10^{-5} mol/L, 15 minutes, n = 5), H-1152 (10^{-6} mol/L, 15 minutes, n = 6), and ICI-192605 (10^{-6} mol/L, 20 minutes, n = 4). B, Mean percentage change in diameter to increasing concentration of 15-F_{2t}-IsoP (n = 8) in the absence or presence of Y-27632 (10^{-5} mol/L, 15 minutes, n = 5) and ICI-192,605 (10^{-6} mol/L, 20 minutes, n = 4).

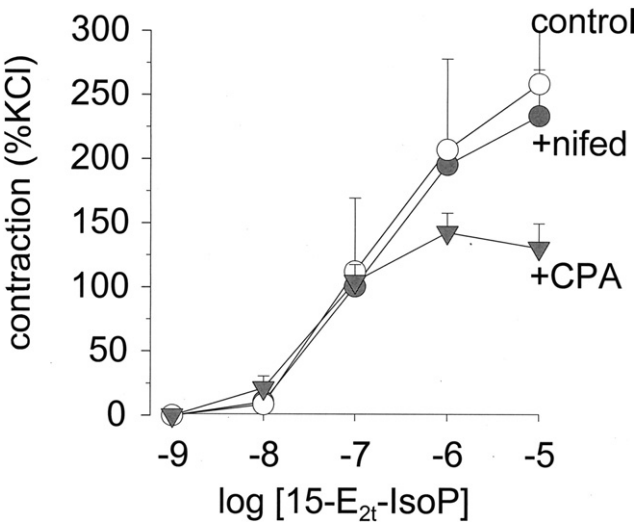

Figure 5. Pharmacologic characterization of the $Ca^{2\pm}$ pools underlying responses to 15-E_{2t}-IsoP. Mean concentration–response relationships for 15-E_{2t}-IsoP–evoked changes in tension in RA rings bathed in the absence or presence of nifedipine (10^{-5} mol/L; n = 7) or cyclopiazonic acid (10^{-5} mol/L; n = 7). *CPA,* Cyclopiazonic acid; *KCl,* potassium chloride.

found the arteries to be highly responsive to the E-ring isoprostane 15-E_{2t}-IsoP and approximately 10-fold less responsive to 2 other structurally similar isoprostanes: 15-F_{2t}-IsoP (possesses a hydroxyl group on the central carbon ring instead of a ketone group) and 15-E_{1t}-IsoP (possesses 1 double bond in the fatty acid side chain rather than 2). This high degree of selectivity among the isoprostanes is suggestive of a receptor-mediated mechanism. We found the isoprostane-evoked response to be abolished by the TP-receptor blockers ICI-192605 and GR 32191A.

We also probed the signaling pathways underlying these responses. TP receptors can mediate altered Ca^{2+} sensitivity via the RhoA/ROCK signaling pathway, as well as release of internally sequestered Ca^{2+}.[7] We found 15-E_{2t}-IsoP responses to be highly sensitive to the ROCK inhibitors Y-27632 and H-1152.[19] Although recent studies showed that Y-27632 can inhibit some protein kinase C isoforms,[20] this is not true of the novel and more selective ROCK inhibitor, H-1152.[21] We also found 15-E_{1t}-IsoP evoked an increase in $[Ca^{2+}]_i$. Although it also partially suppressed K^+ currents, which might have a depolarizing influence on the membrane, the contractions were not sensitive to a blocker of voltage-dependent Ca^{2+} channels (nifedipine). On the other hand, the contractions were suppressed by an inhibitor of the internal Ca^{2+} pump (cyclopiazonic acid). We conclude that this E-ring isoprostane stimulates human RA through TP receptors coupled to release of internally sequestered Ca^{2+} and activation of the RhoA/ROCK signaling pathway.

These data are relevant to currently used clinical practice in CABG surgery vis-a-vis the use of pharmacologic treatments to reduce graft vasoreactivity with the intent of preventing postsurgical vasospasm. A standard approach is to pretreat the graft materials with known vasodilators, such as Ca^{2+} channel blockers (eg, verapamil), nitric oxide donors (eg, nitroglycerine), or papaverine (mechanism of action is poorly understood). However, none of these are irreversible: These are easily diluted from the graft material once coronary blood flow is reestablished and/or are metabolized to less reactive or nonreactive derivatives. Given the important

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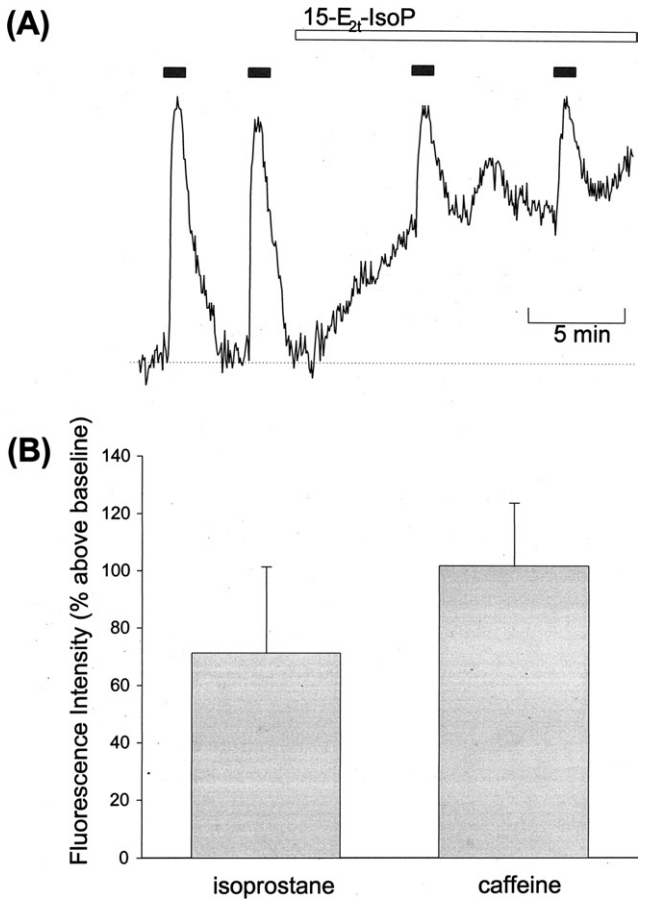

Figure 6. Fluorimetric responses to 15-E_{2t}-IsoP. A, In an enzymatically dissociated human RA cell loaded with the Ca^{2+}-indicator dye fluo-4, 15-E_{2t}-IsoP (10^{-5} mol/L) elevated $[Ca^{2+}]_i$ in a concentration-dependent manner. B, Mean changes in fluorescence (percent above baseline) in response to 15-E_{2t}-IsoP (10^{-5} mol/L in perfusion medium) or caffeine (10 mmol/L, applied by pressure ejection from micropipette).

role played by the sympathetic innervation in control of vasomotor tone, it was thought that treatment of the graft material with adrenoceptor blockers such as phentolamine mesylate (Regitine; Novartis, Basel, Switzerland) or phenoxybenzamine might be beneficial.[22-26] We confirm here that any effect of pretreating the human RA with verapamil and nitroglycerine is easily and quickly lost, whereas pretreatment with the irreversible α-blocker phenoxybenzamine seems to be somewhat more persistent, lasting at least 1 day and several washes. However, this study nonetheless shows that phenoxybenzamine-pretreated vessels can still respond to other non-adrenergic excitatory stimuli: Stimulation of TP receptors with isoprostanes evoked substantial contractions (in fact, even larger than those evoked by α-adrenergic stimuli) irrespective of whether the tissues had been pretreated. Isoprostanes are produced in large amounts by peroxidative attack of lipid membranes and have been shown to accumulate to substantial levels after a wide variety of conditions of oxidative stress,[7,27] including CABG surgery.[8-10] They can be released immediately during the period of oxidative stress or formed while still esterified within the plasmalemma and be released at a later time by phospholipases.[28] Also, additional isoprostane production and release can occur days or weeks after surgery if perfusion is not optimal and oxidative stress recurs. Given that the heart and the tissue graft itself are the source of these isoprostanes, local intra-tissue concentrations would likely be much higher than those measured in the circulating blood. This may account for the postsurgical spasm sometimes seen after such surgeries.

A large body of data attest to the importance of voltage-dependent Ca^{2+} influx in some aspects of vascular smooth muscle reactivity, and oral Ca^{2+} channel blockers remain a primary agent for the prevention of postoperative RA spasm. However, the effectiveness of intraoperative topical

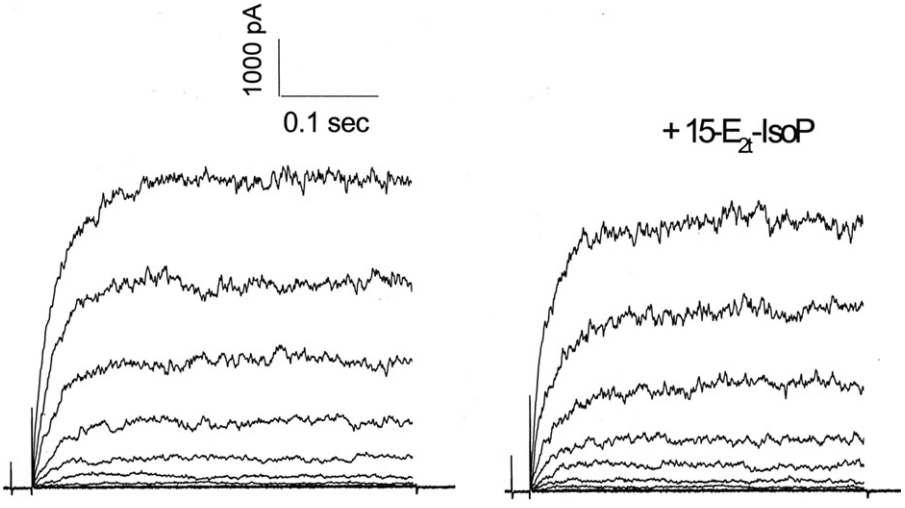

Figure 7. Electrophysiologic response to isoprostane. In a freshly enzymatically dissociated human RA cell, step depolarizations (−60 mV to + 50 mV, in 10-mV increments, from a holding potential of −70 mV) evoked outward currents (*left*) that were suppressed by 15-E_{2t}-IsoP (10^{-5} mol/L) (*right*).

Ca^{2+}-channel blockade has never been proven. Our finding that nifedipine has little effect against the contractions evoked by stimulation of TP receptors or α-adrenoceptors suggest this approach will have little usefulness in preventing RA spasm. We believe our findings warrant clinical studies that test the usefulness of TP receptor blockers used perioperatively on the graft itself or postsurgically as a rescue therapy once vasospasm manifests itself. These have already been assayed for use in several other clinical conditions, and it is interesting that thromboxane receptor blockers are generally found to be much more effective than thromboxane synthesis inhibitors.[29,30] This difference is not expected if thromboxanes are involved but is entirely consistent with isoprostane pharmacology. Alternatively, our data also indicate the potential usefulness of ROCK inhibitors, which would also be effective against the spasmogenic actions of other agonists (eg, endothelin and angiotensin) and may increase the activation[31] and expression[32] of endothelial nitric oxide synthase.

Our data also document that pretreatment of the graft materials with phenoxybenzamine completely abrogates their responsiveness to adrenergic agonists even days after pretreatment and after numerous washings. This finding would suggest that adrenergic agonists can in fact be used postsurgically to elevate overall systemic blood pressure (eg, as an inotrope in the event of shock), because the graft will be unable to respond while the peripheral vasculature exhibits the normal vasopressor response.

This study now sets the stage for several novel research directions. First, further investigation into the pharmacokinetics of thromboxanes and isoprostanes after CABG is needed. Also, the efficacies of alternative approaches to the perioperative pharmacologic treatment of the graft materials and management of the patient need to be evaluated, and other interventions for postsurgical vasospasm should be developed. The surgeon's concern should not be so much toward postsurgical adrenergic responsiveness of the grafted material but rather to more powerful vasoconstrictor stimuli, such as thromboxanes and isoprostanes.

Conclusions

We find the human RA to be highly responsive to isoprostanes, which act through TP receptors to stimulate the release of internally sequestered Ca^{2+} and RhoA/ROCK activities. Given that isoprostanes can accumulate to substantial levels after CABG surgery,[8-10] it is imperative that strategies aimed at treating or preventing postsurgical vasospasm consider the blockade of this signaling pathway.

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